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GRANT NUMBER DAMD17-97-1-7010

TITLE: Therapeutic Hypothermia Following Traumatic Spinal Injury: Morphological and Functional Correlates

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REPORT DATE: January 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | January 1998 | Annual (2 Dec 96 - 3 Dec 97) |

**4. TITLE AND SUBTITLE**
Therapeutic Hypothermia Following Traumatic Spinal Injury: Morphological and Functional Correlates

**5. FUNDING NUMBERS**
DAMD17-97-1-7010

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200*

The general purpose of experiments carried out during the first year focused on the neuroprotective effects of systemic hypothermia and pharmacological treatments following moderate and severe spinal cord injury. In these experiments moderate hypothermia was initiated 30 minutes versus 2 hours post-injury. In anticipation of future therapeutic applications of combined treatment protocols a second purpose of experiments during the first year was to determine the neuroprotective effects of various neurotrophic factors and cytokines. Finally, efforts were made to establish the relationship between epidural and systemic (rectal) temperature prior to and post-injury. The major findings of these studies have shown that significant neuroprotection can be achieved with moderate systemic hypothermia or intraparenchymal infusion of bFGF following moderate injury. No significant neuroprotective effects were found with hypothermia following severe injury. Furthermore, no significant neuroprotection was achieved in animals when either treatment was started 2-3 hours post-injury. Finally, it was determined that epidural temperature was consistently higher, i.e. approximately two degrees, above systemic temperature and increased two degrees post-injury. The results thus support the original hypothesis of this proposal that it is possible to achieve significant neuroprotection with moderate whole body hypothermia.

**14. SUBJECT TERMS**
Behavior evoked potentials, Temperature, Hypothermia, Hyperthermia, Neuroprotection, Steroids, Spinal injury

**15. NUMBER OF PAGES**
53

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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2/27/98

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# ANNUAL REPORT (1998)

# THERAPEUTIC HYPOTHERMIA FOLLOWING TRAUMATIC SPINAL INJURY

ROBERT P. YEZIERSKI, PH.D.

## INTRODUCTION

The research carried out during the first year of the funding period focused on the effects of spinal cord temperature and cytokines/neurotrophic factors on neurologic outcome following traumatic spinal cord injury. The discussion below addresses: (a) the protective effects of hypothermia in models of CNS trauma and ischemia; (b) recent studies supporting the beneficial effects of modest cooling (1-5°C) in CNS injury; (c) neuroprotective effects of cytokines and neurotrophic factors; and (d) the applicability of these findings to traumatic spinal cord injury (SCI).

**Hypothermia and CNS Protection:** The premis that lowering CNS temperature protects against the detrimental effects of hypoxia and ischemia evolved in the 1950's. From a need to protect nervous tissue during vascular operations where circulation to the brain and/or spinal cord was interrupted hypothermia emerged as an adjunct to conventional therapeutic interventions. The protective influence of moderate and severe hypothermia was first demonstrated in experimental models of both spinal and cerebral ischemia (Beattie et al., 1953; Pontius et al.,1954,1955; Marshall et al.,1956; Rosomoff, 1956,1957), and its beneficial effects were concluded to be secondary to the lowering of cerebral metabolic demands. In early studies Rosomoff (1959) described a canine model of middle cerebral artery occlusion where hypothermic treatment (22-24°C for 1 hour) fifteen minutes after ischemia was protective against neurologic injury and death. Similarly, tolerance to interruption of cerebral circulation in dogs was dramatically increased (threefold) when body temperature was reduced to 23°-26°C (Marshall et al., 1956). Similar observations have been reported in primates subjected to prolonged periods (45-75 minutes) of cardiac arrest (Kopf et al., 1975). These observations lead to the utilization of CNS cooling, albeit on a limited scale, as a form of treatment in pathological conditions such as stroke and spinal cord trauma.

Historically, methods for lowering CNS temperature to protect against the detrimental effects of hypoxia and ischemia were based on the observation that hypothermia reduces CNS metabolic activity and cerebral metabolic rate for oxygen. These effects decrease energy requirements of tissue and increase the period it can survive in an energy deficient state (Rosomoff and Holaday, 1954; Hägerdal et al.,1975; Hägerdal et al.,1978; Kramer et. al, 1968, Michenfelder and Theye, 1970). Hypothermia has also been shown to protect against loss of phosphocreatinine and the accumulation of lactate and NADH following cerebral hypoxia (Michenfelder et al.,1976; Hägerdal et al.,1978). In early studies hypothermia, for example, was found to decrease cerebral blood flow and oxygen consumption proportionally from 25°C to 35°C (Rosomoff and Holaday, 1954, Hägerdal et al., 1975). Systemic hypothermia was also shown to significantly lower the rate of cerebral ATP depletion following interruption of cerebral circulation (Michenfelder and Theye 1976, Kramer et al. 1968). Other beneficial actions of hyperthermia include attenuation of edema and hemorrhage formation that occurs in SCI (Green et al.,1973) and the possible dialysation of toxins secondary to perfusing the cord with hypothermic solutions (Tator and Deecke, 1973).

**Mild Hypothermia Is Neuroprotective In CNS Ischemia:** The observation that modest hypothermia is neuroprotective in CNS ischemia was first demonstrated by Berntman et al. (1981) who observed that 1-5°C decreases in body temperature diminished the loss of ATP and phosphocreatinine, and lessened the degree of

brain tissue acidosis following brain hypoxia. Recent studies in which actual brain temperature was monitored strongly suggest that a drastic lowering of CNS temperature is unnecessary to significantly reduce the degree of tissue damage occurring after brain ischemic injury. It has been documented that modest temperature changes (1-5°C) in models of brain ischemia and trauma can significantly alter the extent of neuronal injury and blood-brain barrier alterations (Busto et al.,1987; Dietrich et al.,1990b; Dietrich et al.,1990a; Dietrich et al.,1991). Furthermore, modest brain hypothermia reduces the release of neurotransmitters, such as glutamate, which can mediate secondary injury processes (Busto et al.,1989). These findings suggest that only modest changes in spinal cord temperature may be needed to lessen the extent of tissue injury following trauma.

**Protective Effects of Local Spinal Cord Cooling**: Based on early studies involving brain injury, hypothermic techniques were modified to provide local cooling to the injured spinal cord. The technique was first successfully applied to experimental spinal cord injury by Albin et al. (1965) who perfused the traumatically injured spinal cord of dogs with cold isotonic saline (5°C) for 2.5 hours post-injury. As a result of this treatment there was a dramatic recovery of neurologic function compared to animals without this treatment. These investigators subsequently demonstrated beneficial effects of local spinal cord cooling (LSCC) in a similar SCI model in monkeys, even when the application of LSCC was delayed for four hours after injury (Albin et al.,1968). Following these observations, numerous experimental SCI studies ensued where animals were successfully treated with local hypothermia (Albin et al.,1965,1968; Ducker and Hamit, 1969; Kelly et al.,1970; Black and Markowitz, 1971; Tator and Deecke, 1973; Campbell et al.,1973; Hansebout et al.,1975; Kuchner and Hansebout, 1976; Eidelberg et al.,1976; Wells and Hansebout, 1978).

In the assessment of beneficial effects of LSCC following SCI a variety of outcome measures have been used: (a) evoked potentials (Thienprasit et al.,1975); (b) degree of hemorrhage and edema formation (Green et al.,1973); (c) motor performance (Hansebout et al.,1975; Ducker et al.,1969; Thienprasit et al.,1975); and (d) histopathological analysis (Green et al., 1973). In many of these experiments LSCC was achieved via perfusion with a cold solution or an epidural heat exchanger and investigators aimed at achieving extremely low temperatures (in the neighborhood of 10-15°C below normal). In these studies spinal cord cooling following injury was found to be neuroprotective even if its application was delayed three to six hours post injury (Albin et al.,1968; Ducker and Hamit, 1969; Kelly et al.,1970; Thienprasit et al.,1975; Wells and Hansebout, 1978). Furthermore, the optimal duration of treatment was reported to be approximately four hours (Wells and Hansebout, 1978). Negative studies of LSCC following traumatic SCI have also been noted (Black and Markowitz, 1971; Howitt and Turnbull, 1972; Eidelberg et al.,1976). It should be emphasized, however, that differences in outcome may be secondary to differences in experimental design among different investigators including: (1) animal species; (2) anesthetic regime; (3) method of injury; (4) administration of other drugs; (5) opening the dura; and (6) techniques used to cool the cord. Additionally, spinal cord temperatures were not monitored in a similar fashion in all studies. Some investigators reported only epidural temperatures, and in large species the temperature gradient from epidural to the anterior column can be significant (Wells and Hansebout, 1978). In spite of the inconsistency of experimental design, lack of proper controls, and variability of injury models, most experimental data strongly support the beneficial effects of LSCC in experimental SCI.

The favorable results in animal experiments led to a limited number of cases where local cord cooling was used in human SCI patients (Selker, 1971; Meacham and McPherson, 1973; Koons et al.,1972; Negrin, 1975; Bricolo et al.,1976; Tator, 1979; Hansebout et al.,1984). The results, however, have been difficult to interpret for a variety of reasons: (1) most investigators report only a small number of cases; (2) controls have not been used in any series; (3) variability in level of injury; (4) results have been generally reported as the number of patients that improved or regained function as opposed to utilizing formal grading methods for measuring outcome; (5) the time interval from injury to application of cooling and duration of treatment have

been highly variable; (6) a combination of different drug treatments (usually steroids) have been utilized in conjunction with LSCC; and (7) medical causes of spinal compression other than acute SCI, have been included in some studies. In spite of these complications Hansebout et al. (1984) in reviewing the application of this technique to humans concluded that the results were encouraging. The application of the technique, however, is fraught with technical and logistical difficulties not to mention the clinical challenge of performing a multilevel laminectomy on medically compromised patients (frequently with multiorgan trauma) while trying to minimize the time interval between injury and the application of cord cooling. These obstacles could be overcome if only modest systemic cooling was required in order to produce neuroprotective effects.

The largest patient series using hypothermia was reported by Meacham and McPherson (1973), in which 14 spinal injured-patients were treated with LSCC. Successful initiation of LSCC within 8 hours or less from the time of injury was achieved in all cases and the authors reported return of function in 7/14 patients. A major concern with this study, however, was a mortality rate of 29%, which the authors attributed to the frequency of respiratory complications that occur in cervical injuries. It is difficult to draw definitive conclusions regarding outcome from this study since controls were not utilized and the period of follow-up was not mentioned. Only two other studies contained more than a small number of SCI patients. The first was a series of 11 patients reported by Tator (1979) and the second was a series of 10 patients reported by Hansebout and colleagues (1984). Both studies found that LSCC provided functional recovery in 27% and 43% of patients respectively, which was considered to be higher than would be expected following conventional treatment.

**The Application of Moderate Hypothermia in SCI:** Based on the findings described above, it was hypothesized that mild changes in cord temperature could affect the extent of injury occurring in spinal cord trauma. The rationale for this hypothesis centered around the fact that: (1) local spinal cord cooling has been shown to be beneficial; (2) modest decreases in CNS temperature are effective in models of cerebral and spinal ischemia; and (3) modest decreases in brain temperature in models of CNS ischemia protect against processes which have been implicated in the pathophysiology of SCI, e.g. alterations in the blood-brain barrier, edema formation, production of leukotrienes, and release of neurotoxic substances such as glutamate and aspartate (Dempsey et al. 1987, Dietrich et al. 1990a,b, Busto et al., 1989).

The above observations raised the possibility that modest changes in spinal cord temperature may lessen the extent of tissue injury following trauma. Supportive of this idea Tator and Deecke (1973) reported normothermic perfusion to be as effective as hypothermic perfusion in experimental SCI of moderate severity. The actual temperature of the perfusing solution in these experiments was 36°C, which is mildly hypothermic. Low grade hypothermia is also known to spare ATP and phosphocreatinine concentrations, and decrease the magnitude of lactate accumulation after cerebral hypoxia (Berntman et al.,1981). Finally, systemic hypothermia has been shown to be neuroprotective in an experimental model of spinal ischemia (Robertson et al.,1986). Total body hypothermia in fact has been shown beneficial and is the standard of prophylaxis against ischemic SCI during aortic cross-clamping and cardiothoracic procedures involving controlled cardiac arrest. Its implementation in the treatment of brain injury in large trauma centers has been promising. Once parameters for optimal efficacy have been established in spinal injury, it seems logical that clinical trials in spinal cord hypothermia will offer similar rewards. If hypothermia does provide important clinical benefits, it is of equal importance to the spinal injured patient to determine whether elevated systemic temperatures, as might be experienced during an episode of post traumatic fever, exacerbate the injury process and are detrimental to the recovery of function.

To investigate these hypotheses a preliminary study of modest hypothermia in a weight drop model of SCI in the rat was carried out (Martinez and Green, 1992). In this study female Sprague-Dawley rats (250-300g) were subjected to a 50 gram-centimeter (10 gram weight dropped 5cm) lesion at T8 under halothane-nitrous

oxide anesthesia. Epidural temperature was maintained at 33°C in the first group of animals (n=3), and at 37°C in a second group (n=3). These temperatures were achieved by lowering systemic (rectal) temperature to 31-32°C in the hypothermic animals or raising systemic temperature between 38°C-39°C in normothermic animals. The epidural temperature in each group was maintained for four hours post trauma. Following injury, animals were kept under nitrous oxide anesthesia until the termination of the four hour treatment period. Three days post-injury animals were sacrificed and the spinal cords removed for histological examination. All animals remained completely paraplegic during this observation period. Morphological evaluation at the epicenter of lesion sites, however, revealed that the 38°C animals had significantly more hemorrhage and parenchymal damage than the 32°C animals (Martinez and Green, 1992).

In conclusion, local spinal cord cooling has been shown to be effective in the treatment of experimental SCI. Similar beneficial results have been reported in some clinical studies, but the number of patients is small and controls have not been utilized. In addition, the high mortality reported in some studies remains a major concern with its clinical application. Recent findings of the neuroprotective effects of modest hypothermia in brain ischemia, however, may be applicable to SCI and offer a treatment protocol with fewer complications. Indeed, preliminary observations suggest that modest temperature changes, such as can be produced via systemic hypothermia, can affect the degree of tissue injury following spinal trauma (Martinez and Green, 1992). The importance of such findings is that, compared to LSCC, systemic hypothermia provides a much simpler approach by which the cord can be "cooled" and thus obviates the need for acute surgical intervention. If effective, modest systemic hypothermia would provide an additional therapeutic approach that could be applied to the clinical treatment of acute SCI. Of equal importance hypothermia could be used in neurosurgical procedures of the spinal cord and vascular surgical procedures in which spinal cord perfusion may be compromised. Based on the above discussion it can therefore be concluded that there is sufficient justification in both the scientific and clinical literature for additional studies related to better defining the optimal parameters for the hypothermic treatment of the injured spinal cord.

**Neuroprotective Effects of Cytokines and Neurotrophic Factors:** The initial trauma induced by injury together with the complex cascade of events following injury determines the degree of total tissue damage and the ultimate neurological outcome following SCI. Presently, numerous agents are proposed to be neuroprotective against CNS injury (for recent reviews see McIntosh, 1993; Mattson and Scheff, 1994; Mocchetti and Wrathall, 1995). Steroids, neurotrophins, cytokines, and gangliosides have been demonstrated to promote neuronal survival or support neuronal growth in various *in vitro* systems (Mattson and Scheff, 1994; Blottner and Baumbarten, 1994; Olson et al. 1994.). Methylprednisolone improves neurological recovery when given early after human SCI (Bracken et al., 1990). Recently, neurotrophins have also been utilized in several disease models: glial cell-derived neurotrophic factor (GDNF) in Parkinson's disease, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) in Alzheimer's disease, and insulin-like growth factor (IGF-1) in multiple sclerosis (Hefti, 1997). The neurotrophic factor basic fibroblast growth factor (bFGF) has also been reported to be neuroprotective in models of cerebral ischemia and traumatic brain injury (Koketsu et al., 1994; Fisher et al., 1995; Dietrich et al. 1996) and to protect neurons from axotomy-induced death (Peterson et al., 1996). In a model of spinal cord compression, bFGF administered locally at the site of lesion was reported to improve hindlimb function in combination with methylprednisolone infusion (Baffour et al., 1995). Recently Teng et al. (1997) reported basic and acidic FGF to be neuroprotective for cholinergic neurons following contusion injury in the rat.

IL-4 has been shown to be an anti-inflammatory cytokine, regulating neutrophil and monocyte/macrophage functions (Luering et al. 1997; Niiro et al. 1997). Although the clinical significance has not been established, increased levels of IL-6 have been reported in the cerebrospinal fluid of head injury

patients (Relton et al. 1997). IL-1, a pro-inflammatory cytokine, was found to excerbate ischemic brain injury (Relton et al. 1997). In the area of spinal cord trauma, experimental studies are needed to assess the conseqences of neurotrophic growth factor and cytokine treatment on histopathological outcome.

In view of the long term desire to combine treatment modalities, e.g. hypothermia and pharmacological, we have carried out a preliminary study evaluating the neuroprotective properties of various cytokines and neurotrophic factors on histopathological outcome using a well characterized weight-drop device to produce spinal cord trauma (Appendix manuscript). We utilized a continuous intramedullary infusion system to reliably deliver various factors directly into the site of injury. Our results indicate that the intramedullary infusion of bFGF, NGF, IL-4 or CNTF initiated one hour after moderate SCI significantly reduces contusion volume.

## HYPOTHESES AND TECHNICAL OBJECTIVES

The experiments proposed in the original proposal were aimed at addressing a number of interrelated hypotheses focusing on defining the optimal hypothermic parameters required to produce neuroprotection in the injured spinal cord.

1. **Hypothesis**: There is an interdependent relationship among systemic, epidural, and spinal cord temperatures that can be defined in order to determine the degree of systemic and/or epidural temperature required to produce neuroprotection in the injured spinal cord.

2. **Hypothesis**: Due to the influence of temperature on a wide range of important homeostatic mechanisms necessary for maintaining the structural and functional integrity of spinal tissue, it is proposed that increases in systemic or site of injury temperatures (hyperthermia) will accelerate the injury process, whereas reducing the temperature (hypothermia) will result in neuroprotection.

3. **Hypothesis**: There is an optimum time (post-injury) when post traumatic hypothermia of injured tissue produces the greatest benefit and is most effective in reducing behavioral deficits and morphological damage.

4. **Hypothesis**: There is an optimum duration of hypothermic treatment which results in the greatest benefit to neurologic outcome.

5. **Hypothesis**: There is an optimum period post hypothermia during which neuroprotection is maximal.

**MILITARY BENEFIT OF PROPOSED STUDIES:** If modest decreases in spinal cord temperature, achieved by systemic hypothermia or local cord cooling, are capable of reducing the degree of tissue damage following SCI, such a finding would have a significant impact on current protocols used in the treatment of acute spinal cord injury. The military importance of such a finding relates to the fact that in a combat situation there are a limited number of options available for the treatment of spinal injured soldiers. If cooling the spinal cord is a viable treatment option the ability to cool the cord using transcutaneous or systemic hypothermia would provide a much simpler approach in order to gain the benefits of hypothermic neuroprotection. If effective, modest systemic hypothermia would provide a therapeutic treatment which could be applied to the human condition at times when surgical intervention is logistically difficult or when multiple injuries make other modes of treatment

difficult to implement without severely compromising the survival of the patient. Conversely, it is important to determine the detrimental effects of elevated systemic and cord temperatures in exacerbating the injury process. Since fever secondary to pulmonary compromise and stressed immune function is a common finding in traumatically injured patients, it is important to establish the clinical consequences of modest elevations in systemic temperature (in terms of augumented tissue damage). This information is especially important in the design of treatment protocols for soldiers that develop febrile conditions post injury.

**STATEMENT OF WORK: FIRST YEAR:** During the first year of the funding period one of the primary goals was to establish a therapeutic relationship between spinal cord temperature and neurologic outcome following traumatic spinal cord injury (SCI). In the studies carried out efforts were also made to evaluate moderate hypothermia in order to determine if we could achieve neuroprotection following truamatic SCI. Although local spinal cord cooling has been attempted as a form of treatment in experimental and human SCI, most studies have focused on temperature shifts in the range of 15-20°C. Because of the technical difficulties required to achieve these conditions the application of hypothermia as a therapeutic intervention in SCI has been difficult to implement. Recent experimental data, however, suggests that modest changes (1-5°C) in central nervous system (CNS) temperature may positively influence outcome following CNS injury. The specific aim for the past year was, therefore, intended to utilize an established model of contusive spinal injury, i.e. weight drop, and evaluate the effects of hypothermic treatment on a morphological endpoint following injury. Since we would like to ultimately evaluate the neuroprotective effects of combination therapies, e.g. hypothermia with pharmacological treatment, we also initiated a study to evaluate the neuroprotective effects of neurotrophic factors and cytokines.

1. Initial experiments carried out during the first year focused on defining the relationship between epidural and systemic (rectal) temperatures in uninjured animals. Knowledge of this relationship is necessary to adequately plan subsequent studies where: (1) epidural temperature will be used as an index of spinal cord temperature; and (2) systemic cooling will be used as a method to achieve therapeutic temperatures (in the cord) required to produce neuroprotection.

In these studies we hypothesized that there is an interdependent relationship between epidural and spinal cord temperatures that can be defined in order to determine the degree of systemic and/or epidural cooling required to produce neuroprotection within the injured spinal cord.

In this evaluation we determined that epidermal temperature is slightly higher than rectal temperature (Appendix Figure 1) in the normal rat. Furthermore this relationship was maintained following injury although it is important to point out that there was a modest increase in epidural temperature following injury.

2. Based on observations that slight decreases in brain temperature can significantly improve neurologic outcome following ischemic or traumatic brain injury, it was hypothesized that modest decreases (1-5°C) in spinal cord temperature will provide neuroprotection following traumatic spinal injury.

In experiments carried out during the first year, we determined that moderate hypothermia produced significant neuroprotection within the injured cord (Appendix Figure 3). Additionally, it was important to study the effects of hypothermia on different grades of injury. Therefore, efforts were made to determine the effects of temperature (hypothermia) on neurologic outcome in animals subjected to moderate or severe contusive SCI.

During the first year we have shown the neuroprotective effects of moderate hypothermia on mild SCI. During the upcoming year we are planning to further evaluate this effect by looking in more detail at the therapeutic window of this effect and by evaluating the effects of hyperthermia on functional outcome.

**3**. Identify the time period (post-injury) when hypothermia offers the greatest degree of neuroprotection. In these experiments we have studied the time course of hypothermic effects to evaluate the effects of hypothermia delivered at different times post injury.

During the first year we have evaluated the effects of hypothermia delivered 30 minutes and 2 hours post-injury. Although we found significant effects at 30 minutes post-injury there were no effects in our preliminary evaluation of the 2 hour timepoint.

**4**. Determine the optimal duration of hypothermia which results in the most significant neuroprotective effects.

During the first year we have evaluated a duration of 3 hours of hypothermia following moderate and severe traumatic injury. Although this duration was found to be neuroprotective additional time points need to be evaluated.

**5**. Determine the time course of neuroprotection (post hypothermia) when neurologic outcome is most significantly affected. In these experiments to be initiated during the second year the optimal temperature range (#2), application time (#3), and duration of hypothermia (#4) will be used in evaluating the time-frame of optimal neuroprotection resulting from an optimal dose of hypothermia.

**6**. Determine the effects of combining the most beneficial hypothermic regime with a protocol of pharmacological treatment of a neuroprotective cytokine and/or neurotrophic factor. In these experiments to be carried out during the second year of funding the optimal temperature range (#2), application time (#3), duration of hypothermia (#4), and time interval (#5) will be used in evaluating combined therapeutic treatments.

## EXPERIMENTAL DESIGN AND METHODS

### General Methods

Experimental models of spinal trauma: Although many models have been developed for the production of spinal cord injuries in animals, no single model can perfectly mimic the human condition. Several models, such as the weight drop or Allen (1911) technique and the aneurysm clip compression technique, have been well characterized and documented to create graded, reproducible, spinal cord injuries in rats (Rivlin and Tator, 1978, Gale et al., 1985, Wrathall et al., 1985, Noble et al., 1985). The weight drop method involves dropping a known weight (usually 10g in rodents) a selected distance. As the height of the drop is increased, more severe injuries are produced. In spite of requiring a laminectomy and several reports of it yielding variable neuropathologic changes, the weight drop model remains an accepted standard technique which closely mimics the biomechanics of the human injury and produces injuries which are morphologically similar to those seen in humans (Jellinger, 1976). Furthermore, it has been shown that under careful control of experimental variables, this model will yield reproducible graded injuries (Gale et al., 1985, Wrathall et al., 1985, Noble et al., 1985). In the present studies injuries were produced using a weight drop device obtained from Dr. Wise Young at New York University. This device is presently being used to produce a standard injury in a multi-center study designed to evaluate the

clinical efficacy of drug actions in the treatment of acute spinal cord injury. The injury model to be used is therefore one that has widespread use in the field of spinal cord injury. This feature offers significant advantages, i.e. standardization, when comparing results obtained with different putative therapeutic interventions, e.g. hypothermia versus drug treatments.

Surgical Preparation: Anesthetized adult (250-275g) female Sprague-Dawley rats had the ventral aspect of their neck and back shaved and scrubbed with betadine solution. Level of anesthesia was assessed by monitoring arterial pressure, corneal reflex, and hindlimb withdrawal to noxious stimuli. Using aseptic techniques, PE catheters were placed in the external carotid artery for blood pressure and heart rate monitoring, and in the external jugular vein for fluid and drug administration. Rats were orotracheally intubated with a PE 220 catheter, paralyzed with pancromonium bromide (0.6 mg i.v. followed by a 0.1 mg/kg/hr infusion), and artificially ventilated (Ugo Basile rodent respirator). An anesthetic regime of Halothane, oxygen, and air (see below) was adjusted to maintain physiologically normal levels of $pO_2$ and $pCO_2$. Arterial blood gases were measured every hour with a blood gas analyzer (Radiometer ABL330; 75µl samples). Animals were paralyzed in order to: (1) fully control an animal's respiration and eliminate hypercarbic and hypoxemic effects of anesthetic agents; and (2) ensure physiologically normal blood gases and therefore mimic conditions as they occur in humans at the time of injury.

Contusive spinal cord injury: For producing traumatic spinal injury, a T-8 laminectomy was performed and animals positioned in the weight-drop apparatus as described by Noble and Wrathall (1987). The severity of injury was varied by adjusting the height of the weight drop (10g weight) as follows: mild injuries (2.5 cm), moderate (5-7.5 cm), and severe (12.5-15.0 cm). As mentioned above the weight drop apparatus used was obtained from Dr. Wise Young and is presently the apparatus of choice in a multi-center trial evaluating the effects of drug treatment on acute spinal cord injury. This apparatus is accepted as producing reliable and reproducible injuries of mild, moderate or severe magnitude. The fact that it is being used in studies evaluating potential therapeutic interventions makes it an appropriate choice for use in the present study. Modifications to the parameters (weight and height) used for production of injuries were made based on evaluations of injuries. At the conclusion of surgery durafilm was placed on the surface of the cord, the incision closed in layers, and catheters removed. Post-operatively, animals were housed in cages containing soft bedding and treated with cefazolin (40 mg) i.m. twice a day for 5 days. Water bottles were placed sufficiently low to allow access to water. Food was placed inside the cage until the rats are capable of reaching the standard placement in the cage top. Injured animals were checked daily and bladders palpated at least twice daily and emptied as required until they gain reflex voiding. Body weight was monitored weekly and records kept of all animal care. Antibiotics were administered to animals exhibiting signs of urinary tract infection. Veterinary consultation was obtained for animals demonstrating discomfort or autonomy following injury. In our experience, autonomy was rare in animals with T8 spinal cord injuries. Inclusion criteria for animals to be used in the study consisted of: (a) paraplegia post weight drop; (b) spinal cord hematoma; (c) acceptable weight drop (compression, impact velocity, impact height); and (c) acceptable physiological parameters (blood pressure, pCO2, blood pH).

Measurement and variation of temperatures: Systemic temperature was controlled with a temperature circulator connected to a cooling or warming blanket (Lauda RM6-6 unit which is accurate to 0.1 °C). A flexible thermistor probe (Physitemp IT-21, 410µm diameter) was inserted in the rectum to monitor systemic temperature, and a second thermistor placed laterally at the site of laminectomy in the epidural space to monitor epidural temperature.

Anesthetic Regime: The anesthetic regime consisted of isofluorane (0.5-5.0%), nitrous oxide (20%) and oxygen (20%) in order to produce physiologically normal levels of $pO_2$ and $pCO_2$. The rationale for using this anesthetic combination is: (1) inhalational anesthetics provide a much easier induction, a more uniform level of anesthesia, and a more prompt recovery than injectable anesthetics such as barbiturates or ketamine; and (2) since isofluorane is a commonly used clinical anesthetic this drug regime provides clinical relevance.

Drug Infusion: Cytokines/growth factors: All drugs were purchased from Genzyme Corporation (Cambridge, MA). Interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and ciliary neurotrophic factor (CNTF) were diluted with 0.1M phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA). Two assumptions were made in the calculation of infused drug concentration: 1) the cord is approximately a cylinder with an average cord diameter of 0.4cm and longitudinal lesion length of 0.8cm and the total cord volume to be infused is $0.1cc^3$; and 2) there is a 1:10 dilutional effect by cerebrospinal fluid. The amount of drug calculated for infusion was delivered every day until sacrifice. A 2-10 times excess (based on previously tested *in vitro* concentrations reported by Genzyme Corporation) infusion was achieved for each factor. Control saline (0.1M PBS with 0.5% BSA) was infused in vehicle-treated animals.

Drug Delivery: An osmotically-driven infusion pump (*Alzet minipump*, Alza Pharmacueticals, Palo Alto, CA) was used for continuous infusion of drugs. The pump was connected with PE10/50 tubing to a 30-gauge 3/4 inch needle to deliver drugs through a small dural and pial opening at the site of injury. The cannula was advanced 2mm near the midline at the site of contusion. Cannula insertion and infusion of drugs were started one hour post injury, and continued at 1*ul*/hr for 7 days. The *Alzet* minipump was pre-primed and loaded with drugs, and filled to 225*ul*, including the extra tubing (5 cm). The injection cannula was secured to the preserved lamina below the level of injury and the pump was secured in a subcutaneous pocket with sutures.

Experimental groups consisting of at least 5 rats were compared with vehicle-treated SCI animals. Statistical comparisons were carried out using analysis of variance (ANOVA). The volume of tissue damage, i.e. TZI and ZPP, are presented as mean + standard error, and p-values of $<0.05$ were considered significant (see Appendix manuscript).

Histopathology of Experimental SCI: The morphological changes associated with experimental SCI have been documented in a variety of species (Allen, 1914, Ducker et al., 1971, Bresnahan et al.,1976, Balentine 1978a,1978b, Noble and Wrathall, 1985). Hours following traumatic SCI hemorrhagic changes progress centrifugally and injured areas coalesce to form an area of hemorrhagic necrosis that extends along the longitudinal axis of the cord in a spindle shaped form (McVeigh, 1923, Ducker et al., 1971, Balentine, 1978a). The acute damage is located more centrally and, depending on the severity of the injury, it may progress to involve the adjacent white matter (Ducker et al., 1971). White matter changes begin in the areas adjacent to the gray matter and spread outward in a centrifugal fashion (Bresnahan et al, 1976, Bresnahan, 1978). By the end of the first week postinjury, demyelination and cystic degeneration of necrotic areas becomes evident, particularly in more severe injuries (Ducker et al., 1971, Blight, 1985). By four weeks, the cystic cavity is better defined and the surviving white matter displays demyelination and microcysts (Wagner et al., 1978, Noble and Wrathall, 1985). At four months the cyst is surrounded by astrocytic gliosis and the region of injury shows thickening of the dura mater. An increased cellularity of the leptomeninges is apparent, especially in the more severe injuries (Wagner et al., 1978). Although the morphological analysis to be used in the present study was not extended four months, many of the same analytical protocols alluded to above will be used (see below).

Histological evaluation: At the termination of experiments, animals were deeply anesthetized with sodium pentobarbital and perfused transcardially with a solution of 4% paraformaldehyde and 3.6% glutaraldehyde in 0.1 M Sorensen's phosphate buffer. Injured cord segments, along with surrounding normal cord were removed. Tissue representative of different levels of injury were analyzed using a computer based image analysis system (Image I). Areas of injured gray and white matter were compared with normal controls and the overall configuration and volume of the injury were calculated from serial sections. Sections of interest were stained with cresyl violet to examine the degree of tissue damage.

(a) Data Analysis: An important aspect of all experiments was the quantification of results. In order to establish meaningful relationships among different treatment groups, it was imperative to quantify the amount of tissue damage for animals undergoing different treatments. To this end, transverse or horizontal sections were examined with light microscopy and preliminary reconstructions of the area of tissue damage, i.e. neuronal loss, axonal injury, were made with the aid of an overhead projector and camera lucida (using 1X or 4X objectives). This analysis was carried out by an individual "blinded" to the experimental design for tissue being analyzed. The area of maximal gray and white matter damage at the epicenter of injury sites was evaluated using computer aided imagine analysis (Image I, Universal Imaging Corp.). This technique has been used successfully in preliminary studies to quantify the amount of gray and white matter damage resulting from weight drop injury of the rat cord. This method involves the use of 20-30 longitudinal (horizontal) sections. In horizontal sections the rostrocaudal boundaries of tissue damage have been found easy to distinguish due to the presence or absence of inflammatory cells. Using a low power (1X) objective camera lucida drawings are made of the gray matter of sections from the injection block. Each area was then traced onto a digitizing tablet (Summagraphics) interfaced to a MicroVAX computer system, which computes areas at each horizontal level. The total necrotic area was derived by means of numerical integration of sequential areas. Based on preliminary results this method has provided an effective approach to quantitatively describing the region of tissue damage resulting from weight drop injury in the rat.

## Experiment 1

Specific Aim: To determine the relationship between systemic and epidural temperatures in uninjured animals. Core (systemic) and spinal cord (epidural) temperatures were measured in an effort to establish the interdependence of these temperatures, relationships that will be important to the design experiments 2-3.

Rationale: In order to evaluate the therapeutic benefits of hypothermia in non-invasive treatment protocols it is important to determine the relationship between systemic and spinal cord temperatures.

Experimental Protocol: Rats were anesthetized, intubated, paralyzed and artificially ventilated. Heart rate and blood pressure were monitored in all animals throughout the length of the experiment and $pO_2$ and pCO$_2$ maintained within normal physiologic limits. Blood gases were evaluated every 30 minutes. Systemic temperature was measured by a teflon thermocouple probe inserted in the rectum. Epidural temperature was measured with a teflon thermocouple flexible probe. After a 30 minute control period epidural temperature was lowered by placing the animal in a plexiglas box with circulating thermal blanket. Systemic temperature was monitored continuously along with epidural temperature. The goal of this experiment was to determine the relationship between systemic and epidural temperatures within the range of 32-36°C (systemic temperature).

Results: During these experiments it was found that epidural temperature was slightly higher than rectal temperature. This was found to be the case when systemic temperature was at normothermic (36°C) levels (Figure 1) as well as when systemic temperature was lowered to hypothermic levels (32°C) (Figure 2). This relationship between epidural and systemic temperature is important as it provides information that will be helpful in the design of studies where we will be evaluating specific target temperatures for cord cooling. Knowing the systemic temperature that one needs to obtain in order to achieve a specific epidural temperature will be necessary in order to evaluate the effects of different hypothermic conditions on morphological and functional outcomes.

## Experiment 2

Specific Aim: The objective of this experiment was to study the effects of post traumatic temperature manipulations on neurological outcome in animals subjected to traumatic SCI using mild and moderate contusive injury, and epidural temperatures of 32°C. Neurologic outcome was evaluated using morphological analysis of the injured cord.

Rationale: Using epidural cooling and the relationship determined in Experiment 1 the purpose of these experiments was to evaluate the question: 'will modest hypothermia reduce the severity of spinal cord injury resulting from contusive injury?' Our hypothesis, from work cited in Background (above), is that modest lowering of cord temperature will lessen an animal's functional deficits and structural damage following injury.

Experimental Protocol: Rats were anesthetized, intubated, paralyzed, and artificially ventilated. Heart rate and blood pressure were monitored throughout the length of experiments and $pO_2$ and $pCO_2$ maintained within normal physiologic range. Epidural temperature was changed to maintain a temperature of 32°C (systemic temperature 30°C). Epidural temperature was monitored with a flexible thermocouple probe and contusive lesions produced by the weight drop technique (NYU impactor). Epidural temperature was lowered 30 minutes after injury and maintained at the same level for four hours. The time post-injury for application of hypothermic treatment along with the duration of treatment were both kept constant in this series of experiments. At the conclusion of experiments animals were deeply anesthesized with sodium pentobarbital and following fixation, spinal cord tissue was removed and prepared for histological examination, including determination of the volume of tissue damage.

Results: It was found that significant neuroprotection was achieved when the condition of moderate hypothermia was used following moderate spinal cord injury (Figure 3). No effects of hypothermia were observed under the conditions of these experiments following severe SCI. The results of this study showed that moderate hypothermia for 4 hours was capable of producing a decrease in contusion volume. The fact that there were no significant effects with severe injury indicates that for more severe injury it will be necessary to extend the period of hypothermia and/or increase the level of hypothermia (lower than 32°C). This latter condition is problematic since a hypothermic condition of this level will require a systemic temperature of less than 30°C which is a level that could compromise the survival of traumatically injured animals. It is for this reason that efforts will be made to extend the period of hypothermia (even if it means increasing slightly the epidural temperature to improve animal survival).

## Experiment 3

Specific Aim: To identify the most appropriate time period (post injury) for the initiation of hypothermic treatment.

Rationale: It was hypothesized that there is an optimum time (post-injury) during which hypothermia is most effective in reducing morphological damage following contusive SCI. These experiments are important to establishing the "therapeutic window" during which benefits are derived from hypothermic treatment.

Experimental Protocol: Rats were anesthetized, intubated, paralyzed, and artificially ventilated. Heart rate and blood pressure were monitored in all animals throughout the length of the experiment and $pO_2$ and pCO$_2$ maintained within the normal physiologic range. Contusive injuries were produced by the weight drop technique. In these experiments animals were subjected to moderate injuries at a normal physiologic cord temperature (37°C). Systemic temperature were changed to maintain the epidural temperature at a level of 32°C. This temperature was maintained for a period of four hours. The onset of the hypothermic condition was evaluated at 30 minutes and 2 hours post-injury. Spinal cord epidural temperature was monitored by a flexible teflon thermocouple probe. Following injury and treatment, animals were deeply anesthetized with sodium pentobarbital and transcardially perfused. Following fixation, spinal cord tissue was removed and prepared for histological examination and analysis including calculation of areas of gray and white matter damage.

Results: It was found that when body temperature was lowered commencing within 30 minutes post-injury significant neuroprotection was obtained. By contrast when hypothermia was initiated commencing 2 hours post-injury no effects of treatment were observed for a moderate level of SCI. During the second year efforts will be made to more throughly evaluate the therapeutic window of hypothermic effects against mild and moderate contusion injuries.

## Experiment 4

Specific Aim: The goal of these experiments was to evaluate the neuroprotective effects of neurotrophic factors and cytokines following moderate contusion injury of the spinal cord.

Rationale: Based on the neuroprotective effects of neurotrophic factors and anti-inflammatory cytokines the desire to combine hypothermic and pharmacologic protocols to achieve neuroprotection, we evaluated the effects of intraspinal infusion of cytokines and neurotrophic factors on tissue damage following traumatic SCI.

Experimental Protocol: The present study investigated the neuroprotective effects of interleukins 1 (IL-1), 4 (IL-4), and 6 (IL-6), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF) in a contusion model of spinal cord injury. Female Sprague-Dawley rats (n=55) sustained a 10-gram weight drop injury to the lower thoracic spinal cord (T10) from a height of 12.5mm using the NYU impactor. A micro-infusion system (Alzet minipump) was used to continuously deliver drugs or saline directly into the epicenter of the contused spinal cord starting one or three hours post-injury. At the end of 7 days, animals were perfused for histopathlogical analysis. Longitudinal serial sections were cut on a freezing microtome and stained with cresyl violet. Areas of central necrosis, partial preservation, and total zone of tissue injury were traced by an independent reviewer using a computer based imaging system.

Results: In the vehicle-treated group (n=5), the mean volume of TZI was 18.04 + 1.88mm$^3$ and the mean ZPP was 16.46 + 1.49mm$^3$. The mean injury of different treatment groups are listed in Table 2 (Appendix manuscript). Animals receiving interleukin-1 (IL-1) were found to have consistently larger TZI and ZPP volumes compared to control animals, though the difference was not significant ($p>.05$). It should be noted that despite similar postoperative care, the IL-1 treatment group also had a much lower survival rate (approximately 40%) than vehicle-treated and other treatment groups (approximately 90%). Interleukin 6 (IL-6) did not have any appreciable effects on any of the injury parameters (Figs. 2,3). By contrast basic fibroblast growth factor (bFGF) was the most effective agent in reducing both the TZI ($p=0.0004$, ANOVA) and ZPP ($p=0.0096$, ANOVA) (Appendix manuscript Figs. 2,3). Overall, bFGF reduced TZI by 33% and ZPP by 32% compared to control animals.

Three other drugs were also found to have effects on contusion injury. Ciliary neurotrophic factor, NGF and IL-4 reduced TZI below the control group (ANOVA: CNTF, $p=0.012$; NGF, $p=0.015$; IL-4, $p=0.016$). CNTF infusion had the highest percentage reduction (23%) of these three drugs in terms of TZI (IL-4: 22%; NGF: 21%). Of these three factors, IL-4 reduced ZPP by 20% below control level, CNTF (17%). The reduction of ZPP by NGF was not statistically significant ($p=0.083$).

In an effort to evaluate the potential therapeutic window of drug infusion, bFGF as the most effective treatment was selected for delivery three hours post injury. The comparison of this treatment with vehicle infusion showed no significant reduction in contusion volume (Appendix manuscript Fig. 4).

## CONCLUSIONS AND FUTURE DIRECTIONS

During the first year of funding we have established the relationship between systemic and epidural temperatures. Knowing this relationship will be important as we design experiments to further evaluate the optimum conditions of hypothermia to achieve neuroprotection and to test the effects of different hypothermic and hyperthermic conditions in the second year. We have also determined that there is a critical therapeutic window for the effects of moderate hypothermia delivered for a period of four hours post-injury. In the future we will determine if this window can be extended if the duration of hypothermia is increased for longer periods of time. Since we also determined that moderate hypothermia delivered for four hours does not result in significant neuroprotection on severe injuries, it will be important to determine if this effect can also be improved with longer duration hypothermia. During the second year we would also like to begin exploring the detrimental effects of systemic hyperthermia on neurological outcome. A critically important aspect of our work that will be addressed during the second year is to determine if there are functional correlates to the significant neuroprotection we have observed with hypothermia during the first year.

As one of the long term objectives of our research is the development of a total strategy of neuroprotection following SCI, we have begun in addition to our work with hypothermia to look at possible adjunct therapies that can be added to a hypothermic protocol. To this end, we have determined that significant neuroprotective effects can be obtained with the intraspinal infusion of cytokines and neurotrophic factors commencing within one hour post-injury. No significant differences were observed between animals receiving saline versus bFGF treatment commencing 3 hours after injury. These data demonstrate that the continuous intramedullary infusion of bFGF, IL-4, NGF, or CNTF significantly reduces the total zone of injury, and for bFGF, IL-4, and CNTF the zone of partial preservation after moderate contusion injury of the spinal cord. These results support the further investigation and possible future clinical application of these agents as a solo treatment or in combination with hypothermia as a treatment strategy for acute spinal cord injury.

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# APPENDICES

*FIGURES:*

**FIGURE 1:** Relationship between epidural and rectal temperature under normothermic conditions.

**FIGURE 2:** Relationship between epidural and rectal temperature under hypothermic conditions.

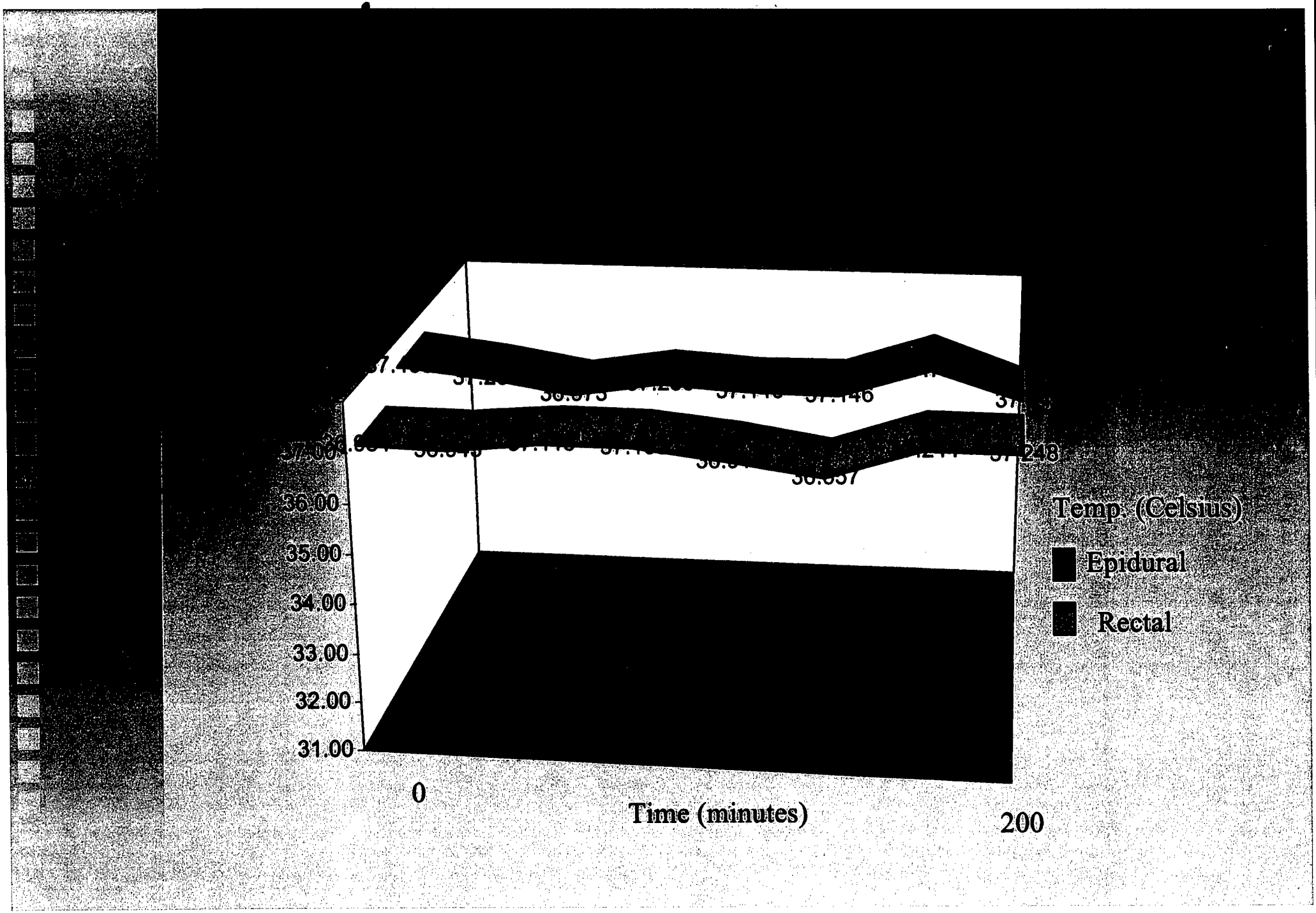
**FIGURE 3:** Neuroprotective effects of mild hypothermia on contusion volume following moderate contusion injury of the rat spinal cord.

*PAPERS:*

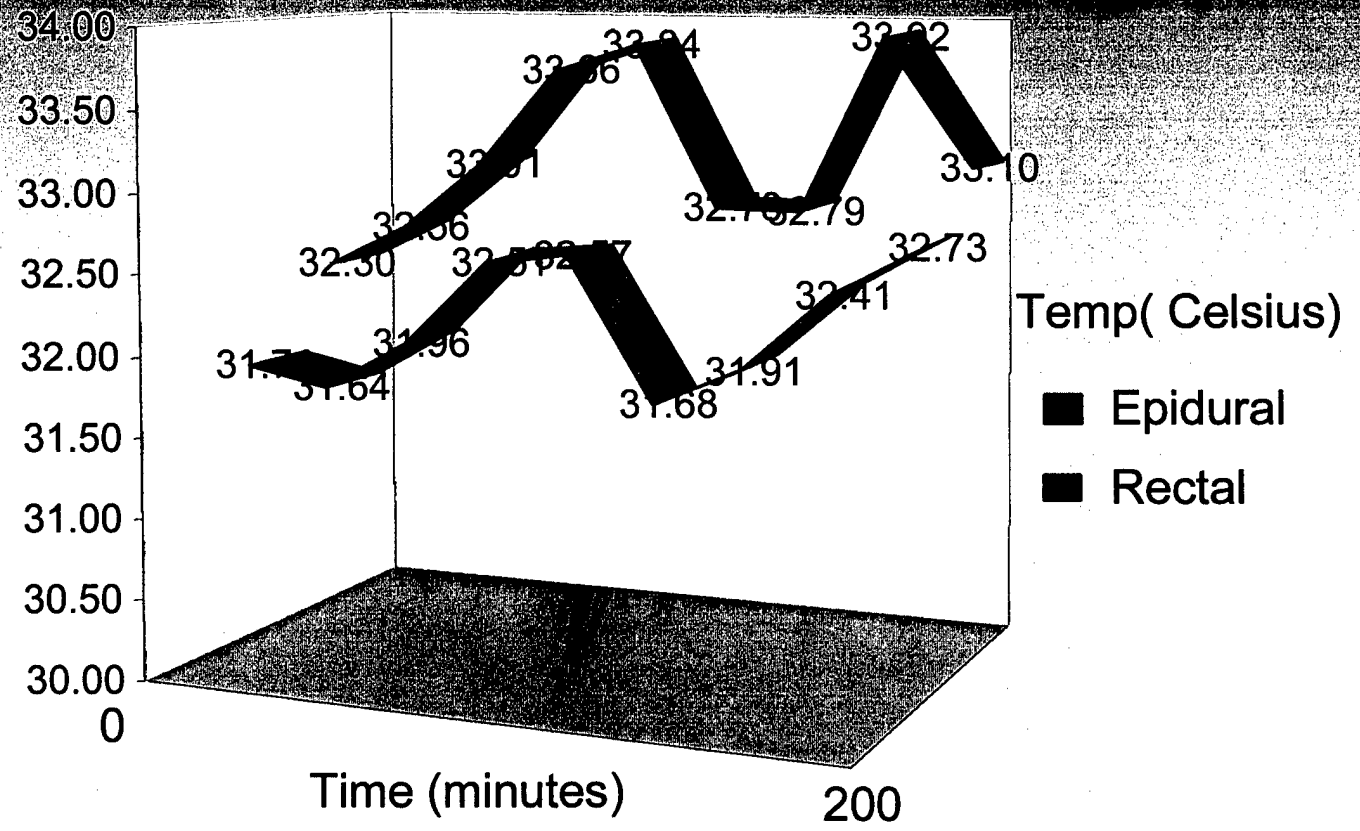
1. Manuscript describing the results of study dealing with the neuroprotective effects of cytokines and neurotrophic factors: Lee et al. J. Neurotrauma (submitted).

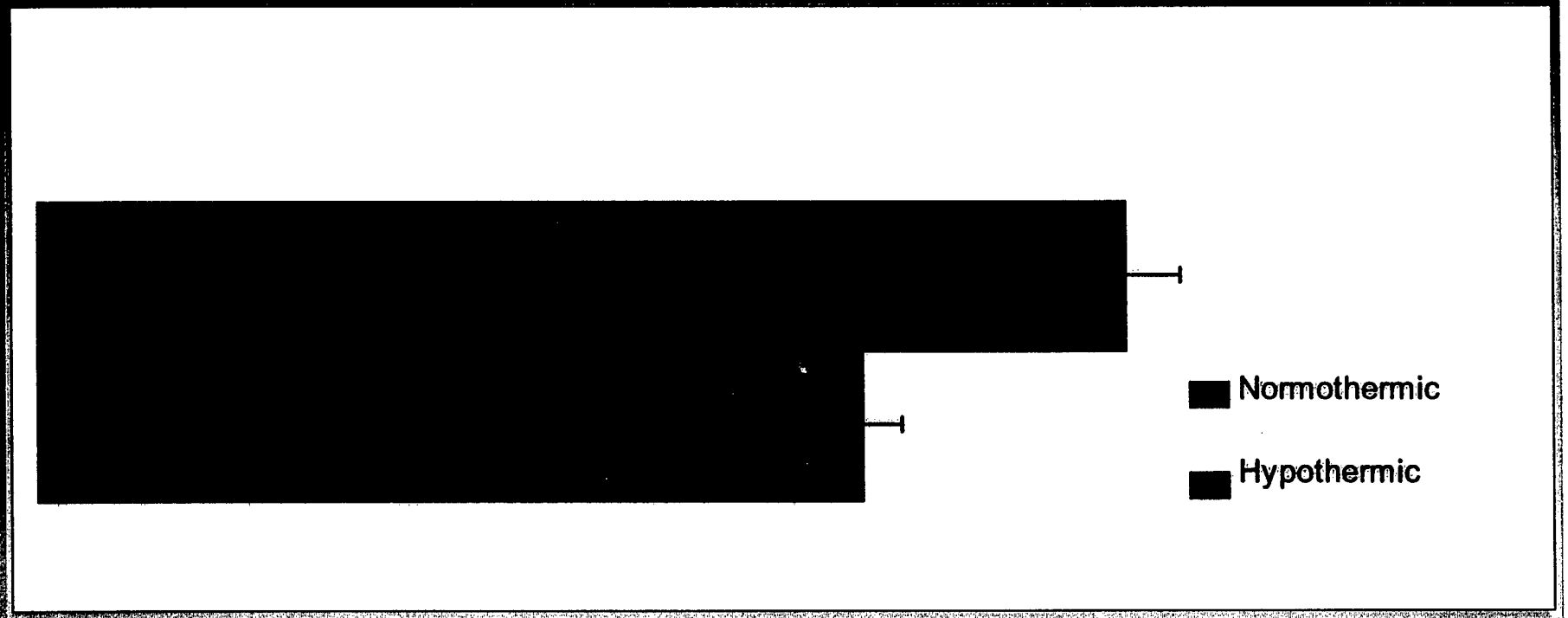
2. Abstract describing the results of systemic hypothermia on acute spinal cord injury: Jimenez et al., J. Neurotrauma 14:772, 1998.

Manuscript describing the results of systemic hypothermia of acute spinal cord injury is presently in preparation.


36.00
35.00
34.00
33.00
32.00
31.00
0
Time (minutes)
200
Temp. (Celsius)
Epidural
Rectal

# FIGURE 2: HYPOTHERMIC EPIDURAL VS RECTAL TEMPERATURE




Normothermic
Hypothermic

# Neuroprotective Effects of Cytokines and Neurotrophic Factors Following Spinal Cord Contusion Injury in the Rat

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**Running Head: Neuroprotection Following Spinal Contusion**

**Key words:** spinal cord injury, cytokine, neurotrophic factor, fibroblast growth factor, interleukin-4, nerve growth factor, ciliary neurotrophic factor

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## ABSTRACT

Cytokines and neurotrophic factors have been implicated in the pathophysiology of brain and spinal cord injury. While some cytokines are considered pro-inflammatory, other factors promote neuronal growth and survival. The present study investigated the neuroprotective effects of interleukins 1 (IL-1), 4 (IL-4), and 6 (IL-6), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF) in a contusion model of spinal cord injury. Female Sprague-Dawley rats (n=45) sustained a 10-gram weight drop injury to the lower thoracic spinal cord (T10) from a height of 12.5mm using the NYU impactor. A micro-infusion system (Alzet minipump) was used to continuously deliver drugs or saline directly into the epicenter of the contused spinal cord starting one or three hours post-injury. At the end of 7 days, animals were perfused for qualitative histopathlogical analysis. Longitudinal serial sections were cut on a freezing microtome and stained with cresyl violet. Areas of central necrosis, partial preservation, and total zone of tissue injury were traced by an independent reviewer using a computer based imaging system. The total zone of injury in 5 animals receiving saline infusion beginning one hour post-injury was $18.04 \pm 1.88 mm^3$. The mean zone of partial preservation in these animals was $16.46 \pm 1.49 mm^3$. Basic fibroblast growth factor reduced the total zone of injury by 33% ($p=0.0004$) in 5 animals and the zone of partial preservation by 32% ($p=0.0096$) when compared to controls. Interleukin-4, NGF, and CNTF also reduced the total zone of injury (CNTF, $p=0.012$; NGF, $p=0.015$, IL-4, $p=0.016$), as well as the zone of partial preservation (IL-4, $p=0.017$; CNTF, $p=0.035$; NGF, $p=0.083$). In contrast, no significant differences were observed between animals receiving saline versus bFGF treatment commencing 3 hours after injury. These data demonstrate that the continuous intramedullary infusion of bFGF, IL-4, NGF, or CNTF significantly reduces the total zone of injury, and for bFGF, IL-4, and CNTF the zone of partial preservation after moderate contusion injury of the spinal cord. These results support the further investigation and possible future clinical application of these agents in the treatment of acute spinal cord contusion injury.

## INTRODUCTION

The initial trauma induced by injury together with a complex cascade of events following injury determine the degree of total tissue damage and the ultimate neurological outcome following spinal cord injury (SCI). Presently, numerous agents are proposed to be neuroprotective against CNS injury (for recent reviews see McIntosh, 1993; Mattson and Scheff, 1994; Mocchetti and Wrathall, 1995). Steroids, neurotrophins, cytokines, and gangliosides have been demonstrated to promote neuronal survival or support neuronal growth in various *in vitro* systems (Mattson and Scheff, 1994; Blottner and Baumbarten, 1994; Olson et al. 1994.). Methylprednisolone improves neurological recovery when given early after human spinal cord injury (Bracken et al., 1990).

Recently, neurotrophins have also been utilized in several disease models: glial cell-derived neurotrophic factor (GDNF) in Parkinson's disease, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) in Alzheimer's disease, and insulin-like growth factor (IGF-1) in multiple sclerosis (Hefti, 1997). The neurotrophic factor basic fibroblast growth factor (bFGF) has also been reported to be neuroprotective in models of cerebral ischemia and traumatic brain injury (Koketsu et al., 1994; Fisher et al., 1995; Dietrich et al. 1996) and to protect neurons from axotomy-induced death (Peterson et al., 1996). In a model of spinal cord compression, bFGF administered locally at the site of lesion was reported to improve hindlimb function in combination with methylprednisolone infusion (Baffour et al., 1995). Recently Teng et al. (1997) reported basic and acidic FGF to be neuroprotective for cholinergic neurons following contusion injury in the rat.

IL-4 has been shown to be an anti-inflammatory cytokine, regulating neutrophil and monocyte/macrophage functions (Luering et al. 1997; Niiro et al. 1997). Although the clinical significance has not been established, increased levels of IL-6 have been reported in the cerebrospinal fluid of head injury patients (Relton et al. 1997). IL-1, a pro-inflammatory cytokine, was found to excerbate ischemic brain injury (Relton et al. 1997). In the area of spinal cord trauma, experimental studies are needed to assess the conseqences of neurotrophic growth factor and cytokine treatment on histopathological outcome.

The purpose of the present study was to determine the effects of various cytokines and neurotrophic factors on histopathological outcome using a well characterized weight-drop device to produce spinal cord trauma. We utilized a continuous intramedullary infusion system to reliably deliver various factors directly into the site of injury. Our results indicate that the intramedullary infusion of bFGF, NGF, IL-4 or CNTF initiated one hour after moderate SCI significantly reduces contusion volume.

## MATERIALS AND METHODS

***Model of Injury***: Adult female Sprague Dawley rats weighing 250-325gms were used in these experiments. All procedures used in this study were approved by the University of Miami Animal Care and Use Committee. Inhalational anesthesia was provided with a balanced halothane, $NO_2$ and $O_2$ mixture. Local anesthetic of 0.25% lidocaine with 1:400000 epinephrine was used to infiltrate the skin and paraspinous muscles. A single dose of intramuscular antibiotic (50mg/kg of cefozalin) was administered at the beginning of the procedure. Aseptic techniques were employed to perform a one level complete laminectomy with bilateral medial facetectomy at the lower thoracic (T10) level of the cord. A 10gm weight drop utilizing the NYU impactor from 12.5mm was performed while monitoring start time, height, and velocity curves. Weight drops with less than 5% height and velocity errors, as well as resultant bilateral cord hematoma and immediate postoperative paraplegia were used as inclusion criteria. Rectal temperature was monitored and isothermic blankets utilized to maintain core temperature at 37°C during the surgical procedure.

***Cytokines/growth factors***: All drugs were purchased from Genzyme Corporation (Cambridge, MA). Interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and ciliary neurotrophic factor (CNTF) were diluted with 0.1M phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA). Two assumptions were made in the calculation of infused drug concentration: 1) the cord is approximately a cylinder with an average cord diameter of 0.4cm and longitudinal lesion length of 0.8cm and the total cord volume to be infused is $0.1cc^3$; and 2) there is a 1:10 dilutional effect by cerebrospinal fluid. The amount of drug calculated for infusion was delivered every day until sacrifice. A 2-

10 times excess (based on previously tested *in vitro* concentrations reported by Genzyme Corporation) infusion was achieved for each factor (**Table 1**). Control saline (0.1M PBS with 0.5% BSA) was infused in vehicle-treated animals.

***Drug Delivery***: An osmotically-driven infusion pump (*Alzet minipump*, Alza Pharmacueticals, Palo Alto, CA) was used for continuous infusion of drugs. The pump was connected with PE10/50 tubing to a 30-gauge 3/4 inch needle to deliver drugs through a small dural and pial opening at the site of injury. The cannula was advanced 2mm near the midline at the site of contusion. Cannula insertion and infusion of drugs were started one or three hours post injury, and continued at 1*ul*/hr for 7 days. The *Alzet* minipump was pre-primed and loaded with drugs, and filled to 225*ul*, including the extra tubing (5 cm). The injection cannula was secured to the preserved lamina below the level of injury and the pump was secured in a subcutaneous pocket with sutures.

***Assessment of injury and protection***: Animals were perfused with 10% formalin on day seven under sodium pentobarbital anesthesia. After fixation cords were removed and placed in 10% sucrose for 24 hours. Cords were cut longitudinally (100*um*) on a freezing microtome. Two-dimensional mapping of the injury site was carried out. Area values were then used to calculate the volume of injury in each experimental group by numeric integration of sequential areas. In preliminary studies, the NYU impactor was shown to produce a reproducible area of spinal cord contusion at 7 days after injury. The well demarcated injury site consisted of an area of central necrosis (CN) defined as the area of cystic degeneration and complete tissue necrosis (**Figs. 1,5**). Surrounding the area of CN was commonly a zone that appeared partially preserved. This zone of partial preservation (ZPP) was defined as the region of vacuolation, selective neuronal injury, and white matter swelling (**Figs. 1,5**). In this study, both the area of central necrosis and total zone of injury (TZI) were measured by an independent blinded reviewer. The zone of partial preservation was calculated as the difference between TZI and CN (**Fig. 1**).

Experimental groups consisting of at least 5 rats were compared with vehicle-treated SCI animals. Statistical comparisons were carried out using a paired t-test. The volume of tissue damage, i.e. TZI and ZPP, are presented as mean ± standard error, and p-values of $<0.05$ were considered significant.

## RESULTS

In the vehicle-treated group (n=5), the mean volume of TZI was 18.04 ± 1.88mm$^3$ and the mean ZPP was 16.46 ± 1.49mm$^3$. Animals receiving interleukin-1 (IL-1) were found to have consistently larger TZI and ZPP volumes compared to control animals, though the difference was not significant ($p>.05$). It should be noted that despite similar postoperative care, the IL-1 treatment group also had a much lower survival rate (approximately 40%) than vehicle-treated and other treatment groups (approximately 90%). Interleukin 6 (IL-6) did not have any appreciable effects on any of the injury parameters (Figs. 2,3). By contrast basic fibroblast growth factor (bFGF) was the most effective agent in reducing both the TZI ($p=0.0004$) and ZPP ($p=0.0096$) (Figs. 2,3). Overall, bFGF reduced TZI by 33% and ZPP by 32% compared to control animals.

Three other drugs were also found to have effects on contusion injury. Ciliary neurotrophic factor, NGF and IL-4 reduced TZI below the control group (CNTF, $p=0.012$; NGF, $p=0.015$; IL-4, $p=0.016$). CNTF infusion had the highest percentage reduction (23%) of these three drugs in terms of TZI (IL-4: 22%; NGF: 21%). Of these three factors, IL-4 reduced ZPP by 20% below control level, CNTF (17%). The reduction of ZPP by NGF was not statistically significant ($p=0.083$). None of the drugs evaluated in this study had a significant effect on the area of central necrosis.

In an effort to evaluate the potential therapeutic window of drug infusion, bFGF as the most effective treatment was selected for delivery three hours post injury. The comparison of this treatment with vehicle infusion showed no significant reduction in contusion volume (Fig. 4). No other drugs were evaluated in this delayed treatment paradigm.

## DISCUSSION

The CNS response to injury uniquely involves the interactions between multiple cell types and injury processes (Hefti, 1997; Hirschberg et al. 1994; Relton et al. 1997). Cytokines and neurotrophic factors have been reported to promote neural growth and protect against ischemic, traumatic, and chemically-induced neuronal damage. These intercellular factors act primarily in a paracrine fashion, with glial and inflammatory cells

producing and secreting them locally (Maeda, 1994). The post traumatic breakdown of the blood-brain-barrier (BBB) also permits the extravasation of blood-borne factors and cellular elements that would be expected to promote inflammatory processes. Thus, two primary factors may potentially act against each other following the initial damage of the central nervous system: a) inflammatory factors which exacerbate damage, and b) the cytokines/neurotrophic factors which promote neuronal regeneration and recovery (Giulian et al. 1988). Certain cytokines may cause further tissue damage by the induction of a surface mitogen mediated immune response, as well as by direct cytotoxicity (Birdsall, 1991). It must be stressed that following injury to the brain or spinal cord, multiple factors affect the cellular response to injury, leading to complex interations that may affect outcome. Thus, although the use of *in vitro* purified cellular systems are a powerful approach in which to investigate the cellular response to injury, experimental models of CNS injury are necessary to determine the potential use of these agents in a clinical setting.

The weight drop system, utilizing the NYU impactor, provides a well characterized model for spinal cord injury. Most spinal cord injuries are blunt in nature, causing parenchymal contusions, hematoma, and edema. In the present study, evidence of a well-defined contusion was seen at seven days after injury. Although the utilization of an injection needle to deliver the agents was invasive, this method assured the direct delivery of the various factors to the injury site. Based on previous data from brain injury studies (Fisher et al., 1995; Dietrich et al., 1997), intravenous infusions may also be considered as a method of drug delivery in the future. However, potential problems including systemic side effects or unpredictable rates of drug delivery to the injury site will have to be evaluated prior to clinical application. Systemic infusion is certainly the easiest in terms of clinical application, and is currently being used in a clinical trial for acute stroke (Dr. Seth Finklestein, personel communication). Intrathecal infusion remains another possibility, although site-directed drug delivery is not achieved with this approach.

Both interleukin-1 (IL-1) and tumor necrosis factor (TNF) are primarily the products of monocyte/macrophage/microglia lineage cells. IL-1 stimulates monocytes and macrophages in an autocrine and paracrine fashion. T and B cell activation is another function of IL-1. IL-1 and its receptors (Yabuuchi etl al.

1994) have been widely characterized and mapped in the brain, and appear to play a significant role in thermoregulation. IL-1 may also regulate neuron-glial, and glial-glial interactions (Hannum et al. 1991). Significant elevations in IL-1 have been demonstrated after experimental ischemia and trauma investigations and following clinical head injury (Relton et al. 1997). In addition, some benefits were reported after brain injury with the administration of the recombinant IL-1 receptor antagonists (Hannum et al. 1990; Relton et al. 1997). IL-1 has been reported to induce intercellular adhesion molecule 1 (ICAM-1) expression and neutrophil-mediated immune responses (Birdsall, 1991). In the present study, a detrimental effect of IL-1 on contusion volume was observed. Thus, the administration of antibodies directed against IL-1, or corresponding receptors may therefore prove to be beneficial in the treatment of acute SCI.

Interleukin-3 (IL-3) and interleukin-4 (IL-4) are T-lymphocyte derivatives and primarily activate B cells (Lee et al, 1993b). Both cytokines have been reported to stimulate peripheral monocyte and microglia growth, and activate ICAM-1 and lymphocyte function associated antigen (LFA-1) on microglial surfaces (Lee et al., 1993b). Possible surface mitogen-mediated cellular and humoral immunity may ensue. IL-4, on the other hand, has been observed to downregulate monocytes and neutrophils (Lugering et al. 1997; Niiro et al. 1997). Their direct effects on neurons have not been fully characterized. In the present study, a neuroprotective effect of IL-4 was documented. This positive effect may be due to the anti-inflammatory nature of IL-4.

Interleukin-6 (IL-6) is produced by monocytes, macrophages, fibroblasts, activated T and B cells, and astrocytes *in vitro* (Lee et al. 1993a). The target cells include pleuri-potential progenitor, B cells, and cytotoxic T cells. IL-6 has been reported to sustain both astrocyte and neuron survival *in vitro* (Kushima and Hatanaka, 1992; Maeda et al. 1994), and appears to interact with a subunit of the ciliary neurotrophic factor (CNTF) receptor (Saad, 1991). Generally considered to be pro-inflammatory, IL-6 have been reported to promote the survival of acetylcholinesterase (AChE)-positive neurons in embryonic rat spinal cord cultures (Kushima and Hatanaka, 1992) and enhance neuronal survival from hypoxia/reoxygenation injury (Maeda et al. 1994). Increased cerebrospinal cord levels of IL-6 have been observed in both adult and pediatric head injuried patients (Bell et al. 1997; Relton et al. 1997). Ras-GTP complex accumulation in pheochromocytoma cell lines was

induced by IL-6 as well (Nakafuku et al. 1992). IL-6 was shown in the present study not to be neuroprotective after SCI. .

Both neutrophins NGF (Blottner and Baumbarten, 1994; Holtzman et al. 1996; Oudega and Hagg, 1996; Saad et al. 1991, Sariola et al. 1994), and CNTF (Blottner and Baumbarten, 1994; Hefti, 1997) trigger neuronal regeneration and induce neuronal differentiation. NGF protects against ischemic brain injury *in vitro* (Holtzman et al. 1996). Its protective action for cholinergic neurons has also been demonstrated (Quirion et al. 1991). The $CNTF_a$ receptor is homologous to the IL-6 receptor (Sariola et al. 1994) in that similar dimerization mechanism of their receptors have been reported. Both have been utilized for the experimental treatment of neurodegenerative disease with variable results (Blottner and Baumbarten,1994; Hefti, 1997). In this study, both CNTF and NGF were demonstrated to be neuroprotective after spinal cord contusion.

Fibroblast growth factor (FGF) (Hefti, 1997; Murphy et al, 1994; Olson, 1994) stimulates neuronal proliferation and sustains its survival, but apparently inhibits differentiation (Murphy et al. 1994). FGF also activates rat pheochromocytoma PC12 cells (Nakafuku et al. 1992), and has been reported to enhance the growth of fetal cerebral cortex, hippocampus, and spinal cord (Olson et al. 1994). FGF is a powerful stimulator of angiogenesis, and promotes wound healing. Recently, Cheng et al. (1996) succesfully utilized acidic FGF (aFGF, FGF-1) as part of the growth medium for interposition nerve grafts after rat thoracic spinal cord transection. Basic FGF or FGF-2 has been reported to be neuroprotective following experimental focal ischemia and traumatic brain injury (Fisher et al., 1995; Dietrich et al. 1996). In recent studies, bFGF together with methylprednisolone treatment was also found to improve behavioral recovery after spinal cord compression (Baffour et al., 1995) and both bFGF and aFGF protect cholinergic neurons following contusion SCI (Teng et al., 1997). bFGF stimulates astrocyte proliferation, and may mediate glial and neuronal interactions important to cell survival (Giulian, 1988). In the present study, bFGF was found to significantly reduce both the total zone of injury, and the zone of partial preservation. Based on these and previous findings, we conclude that bFGF is an important candidate for future investigations directed toward establishing a therapy for acute SCI.

Future experiments could combine various factors, including bFGF, IL-4, and CNTF for simultaneous infusion. In this regard, Balfour and colleagues (1995) have reported a synergistic effect of bFGF and methylprednisolone on neurological function after experimental SCI. Because of the recent success of moderate hypothermia in the clinical treatment of traumatic brain injury, (Marion et al. 1997), combination therapy with mild hypothermia (Jiminez et al., 1997; Martinez-Arizala and Green, 1992) should also be considered following SCI. In the present study bFGF delivered three hours after injury did not exhibit neuroprotection, but further experiments to better delineate the therapeutic window for bFGF treatment need to be performed. Long-term survival with functional and behavioral testing is also required to fully evaluate the benefits of such treatment paradigms. Based on the results of the present study combined with other pre-clinical data, it is proposed that intraspinal delivery of nerve growth factors, together with antiinflammatory cytokines should be considered for future clinical application.

## ACKNOWLEDGMENTS

The authors would like to thank Santiago Castro, Gladys Ruenes, Donald Hesse, Susan Kraydieh, and Dr. Martin Oudega for their technical support, Charlaine Rowlette for word processing and editorial assistance, and Dr. John Bethea for comments on the manuscript. This work was supported by The Miami Project to Cure Paralysis, the U.S. Army (DAMD17-97-1-7010), and NINDS NS-30291.

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## FIGURE LEGENDS

**FIGURE 1** Schematic illustration of spinal cord contusion injury caused by the NYU impactor. TZI = total zone of injury; ZPP = zone of partial preservation; CN = central necrosis. Central necrosis was defined as area of cystic degeneration and tissue necrosis. The zone of partial preservation was defined as the region of vacuolation, selective neuronal injury, and parenchymal edema, though the neuropil was intact and was calculated as the difference between TZI and CN.

**FIGURE 2**: Effects of drug infusion commencing one hour post injury and continuing for seven days on the total zone of injury. Compared to infusion of vehicle (control), IL-1 was found to have a detrimental effect, IL-6 no effect while CNTF, NGF, IL-4 and bFGF significantly reduced the total zone of injury. * = $p < 0.01$; ** = $p < 0.001$.

**FIGURE 3**: Effects of drug infusion commencing one hour post injury and continuing for seven days on the zone of partial preservation. Compared to infusion of vehicle (control), IL-1 and IL-6 were found to have detrimental effects while CNTF, NGF, IL-4 and bFGF significantly reduced the zone of partial preservation. * = $p < 0.05$; ** = $p < 0.001$.

**FIGURE 4**: Effects of bFGF infusion commencing three hours post injury and continuing for seven days. No significant difference was observed between animals infused with vehicle and those receiving bFGF on either the total zone of injury (TZI) or zone of partial preservation (ZPP).

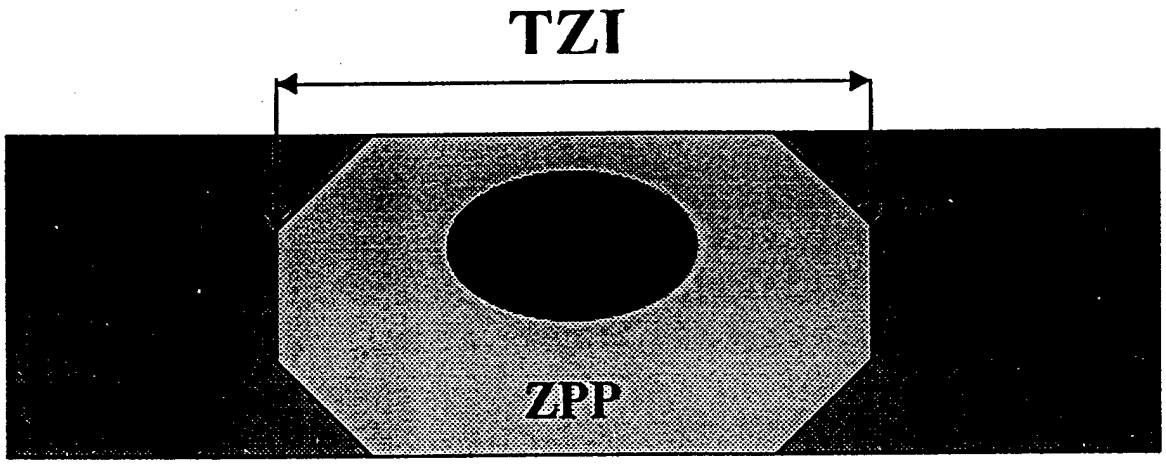
**FIGURE 5**: Cresyl violet stain of comparable sections of contused rat spinal cord after control saline infusion (**A,B**) or bFGF infusion (**C,D**) Note reduced area of injury after bFGF infusion. Scale bar in D equals 915 μm (A,C) and 355 μm (B,D).

**Table 1:** **Dosage of interleukins and growth factors infused into the epicenter of a moderate contusion injury of the rat spinal cord.**

| Factor | Vial | Specific activity | Solvent | Dilution | [Final] |
|---|---|---|---|---|---|
| IL-$1_a$ | 25 ug/ml | $8x10^6$u/mg | PBS+ 0.1%BSA | 1:100 | $2x10^3$u/ml |
| IL-4 | 1.5ug/ml | $5x10^6$u/mg | PBS + 1% BSA | No | $1.5x10^4$u/ml |
| IL-6 | 5 ug/ml | $5x10^6$u/mg | PBS+ 0.1%BSA | 1:20 | $1.25x10^5$u/ml |
| NGF | 20ug/ml | $5x10^5$u/mg | $H_2O$ | No | $2x10^4$ng/ml |
| CNTF | 100ug/ml | $2x10^7$u/mg | PBS+ 0.1%BSA | 1:20 | $5x10^3$ng/ml |
| bFGF | 1mg/ml | N/A | 0.1% CHAPS 0.5% BSA | 1:500 | $10^4$ng/ml |

Table 2. Effect of cytokines and growth factors on spinal cord contusion injury. ZPP was calculated as TZI - CN.

| Drug Infusion | Total Zone of Injury | Central Necrosis | Zone of partial preservation |
|---|---|---|---|
| Control | $3.59x10^8$ $um^3$ | $4.50x10^7$ $um^3$ | $3.14x10^8$ $um^3$ |
| IL-6 | $3.12x10^8$ $um^3$ | $7.26x10^6$ $um^3$ | $3.05x10^8$ $um^3$ |
| IL-1 | $3.74x10^8$ $um^3$ | $1.75x10^7$ $um^3$ | $3.57x10^8$ $um^3$ |
| IL-4 | $2.44x10^8$ $um^3$ | $1.60x10^7$ $um^3$ | $2.28x10^8$ $um^3$ |
| bFGF | $1.97x10^8$ $um^3$ | $1.93x10^7$ $um^3$ | $1.77x10^8$ $um^3$ |
| NGF | $2.46x10^8$ $um^3$ | $5.89x10^6$ $um^3$ | $2.40x10^6$ $um^3$ |
| CNTF | $2.51x10^8$ $um^3$ | $3.93x10^6$ $um^3$ | $2.47x10^8$ $um^3$ |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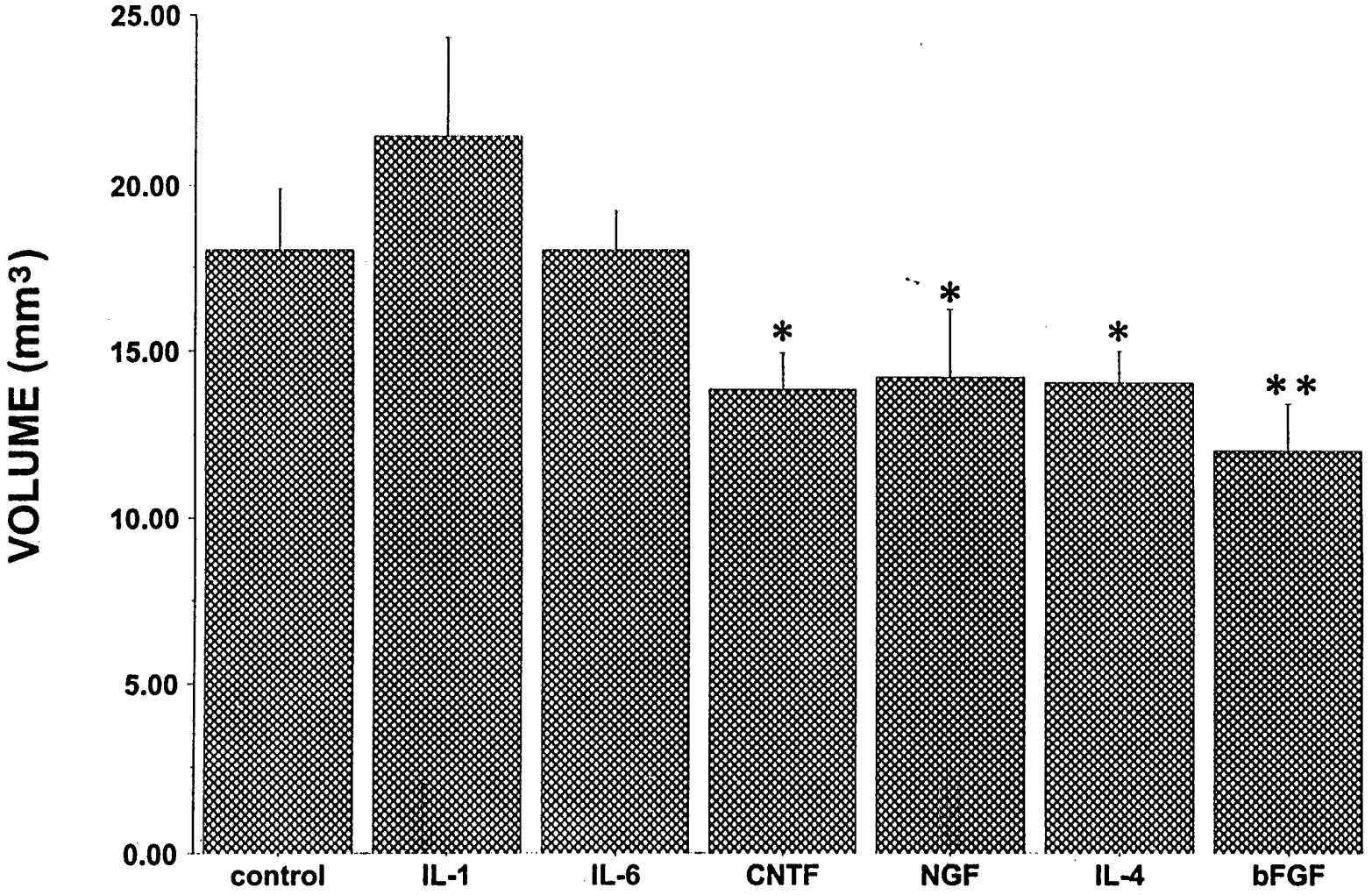
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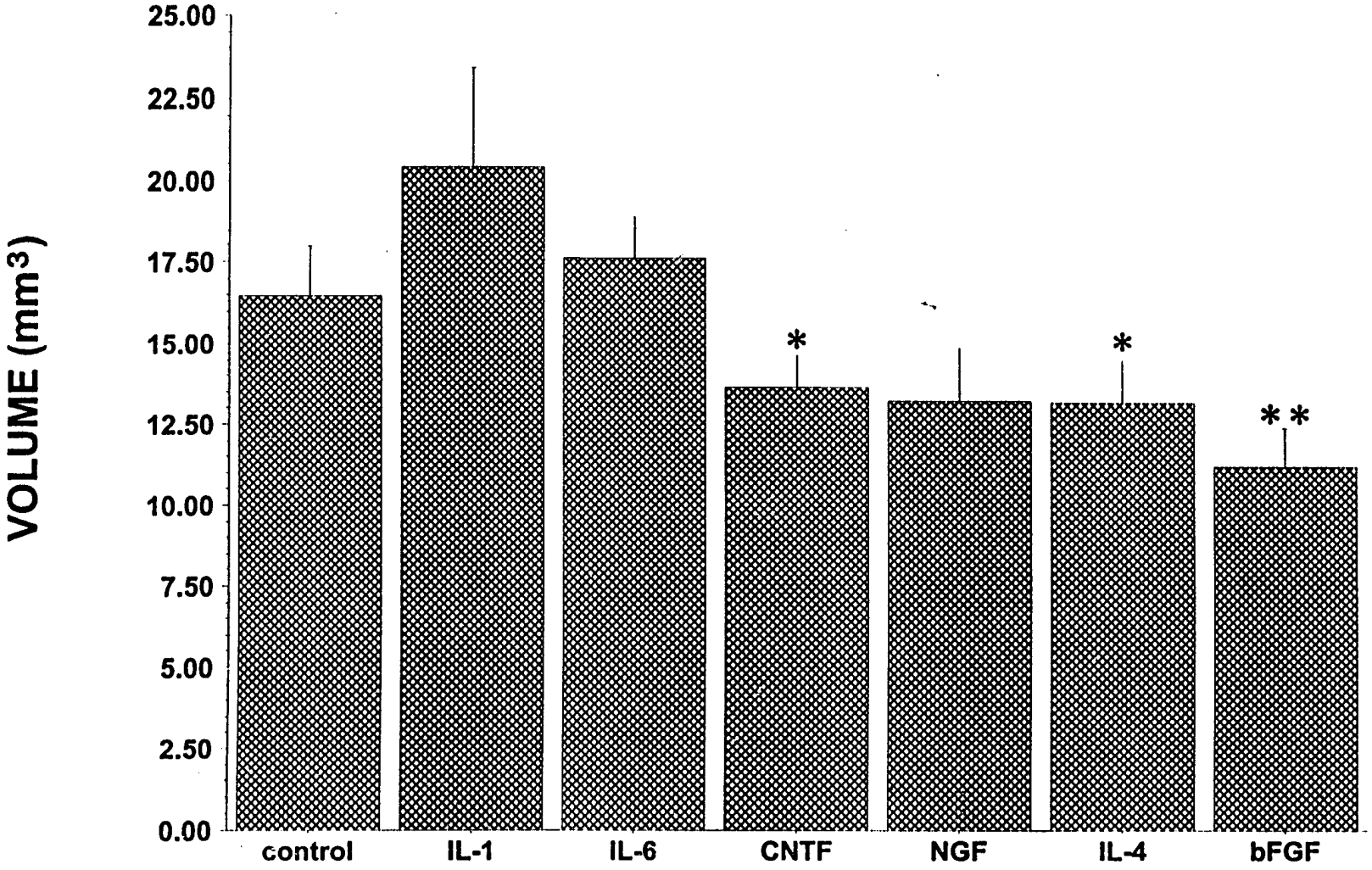
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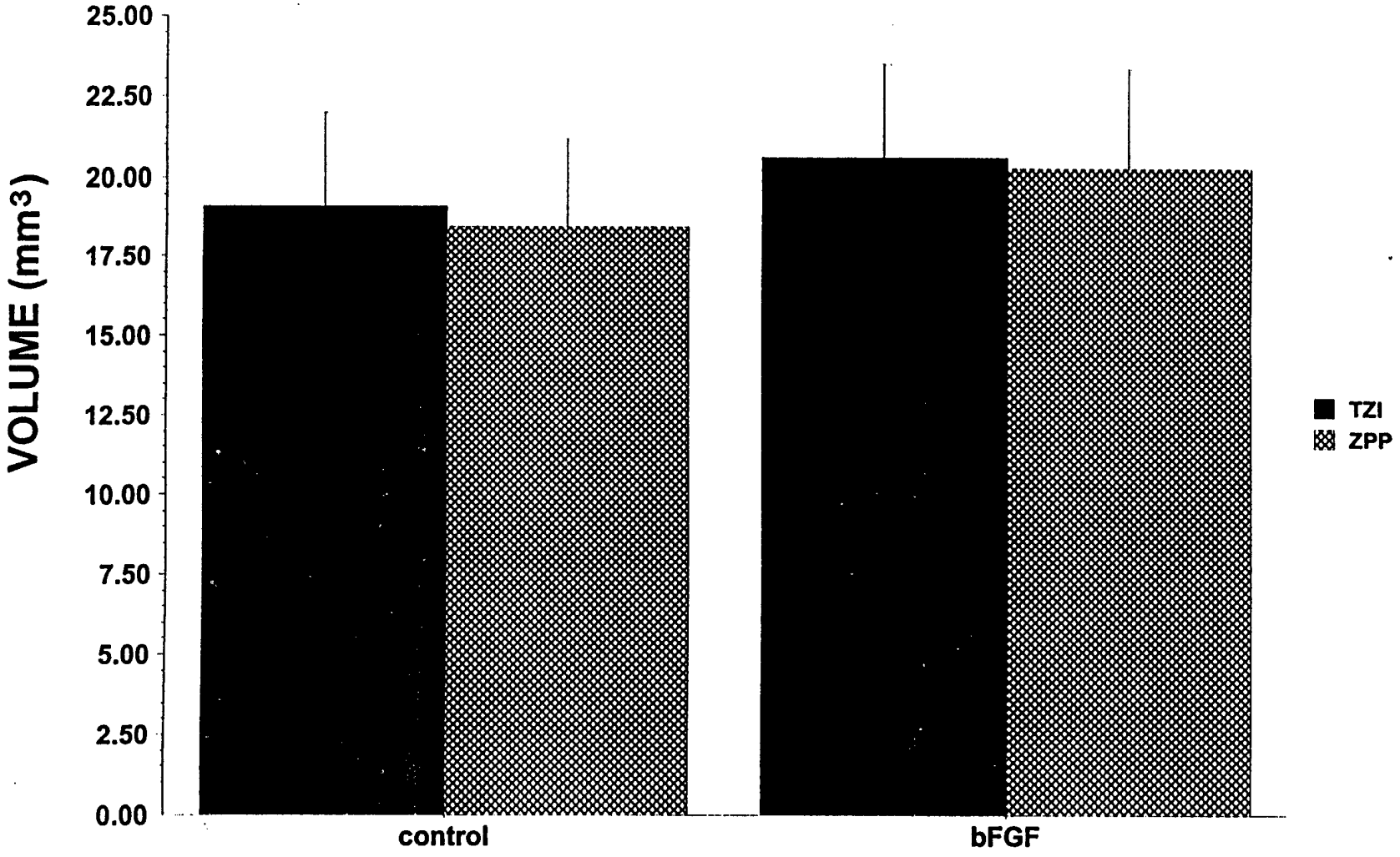
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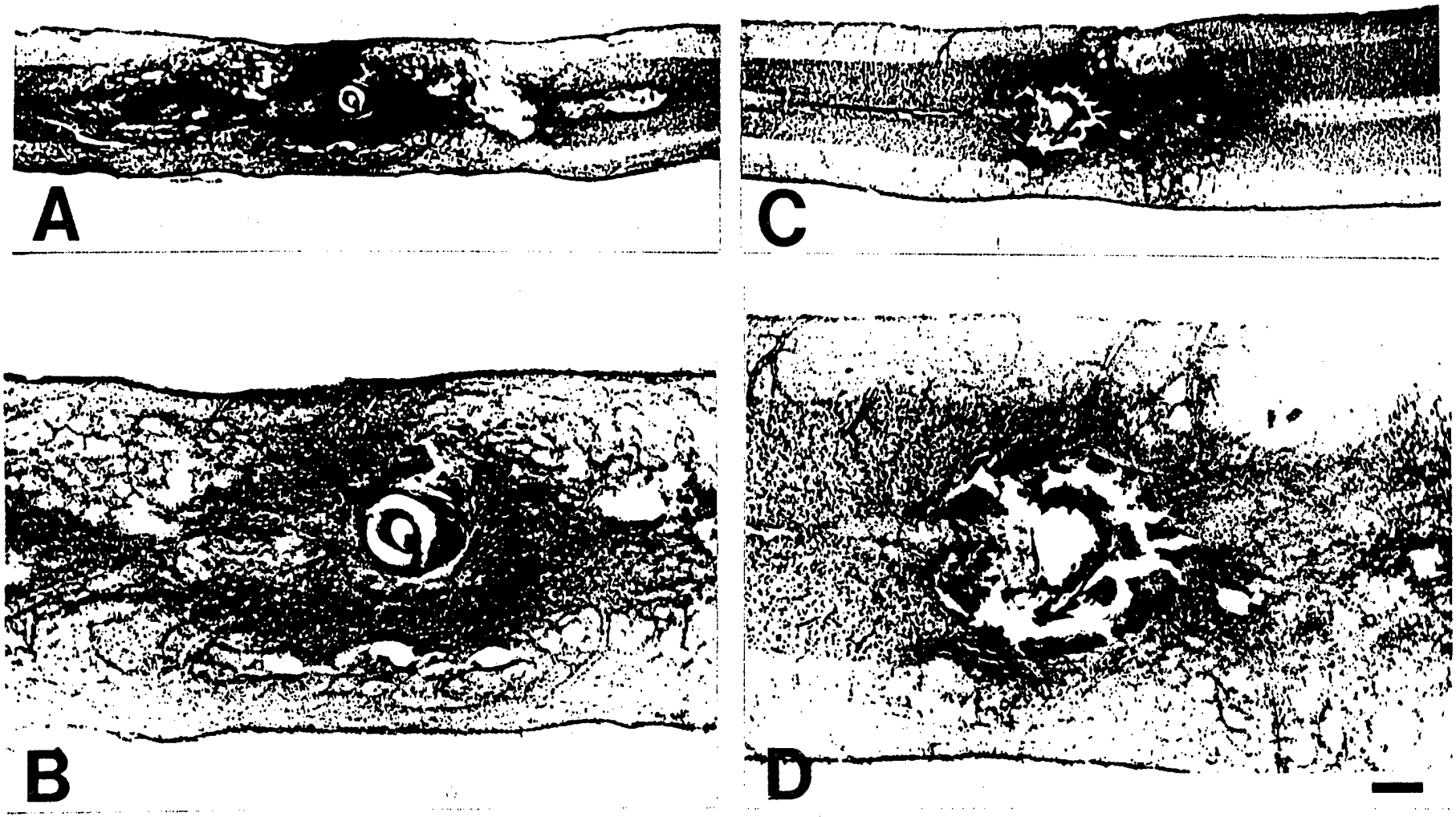
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## EFFECTS OF SYSTEMIC HYPOTHERMIA FOLLOWING ACUTE TRAUMATIC SPINAL CORD INJURY IN THE RAT

O. Jimenez[a*], B. Wieder[a], K. Bangerter[a], W.D. Dietrich[b], A. Martinez-Arizala[bc] and R.P. Yezierski[a,c]
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Local cooling of the spinal cord has beneficial effects following ischemic or traumatic spinal cord injury (SCI). However, local cooling in humans with acute traumatic SCI requires surgical exploration that may be detrimental in a spinal injured and multiple trauma patient. Systemic hypothermia offers an alternative to local spinal cooling. In the present study data are presented supporting the efficacy of systemic hypothermia in traumatic SCI. The weight drop method (N.Y.U. Impactor; 12.5 gram-centimeters) was used in Sprague Dawley rats anesthetized with a mixture of isofluorane-nitrous oxide. Animals were divided into hypothermic (n=5) and normothermic (n=5) groups. Within 30 minutes following injury rats were placed in a plexiglass chamber for 4 hours. Hypothermic and normothermic animals were maintained at an epidural temperature of 32-33°C and 37-38°C, respectively. Blood pressure, blood gases, and temperatures were monitored throughout the 4 hour treatment period. Seven days after injury animals were perfused and the cords quantitatively analyzed. The results showed animals undergoing hypothermic treatment had a significantly smaller volume of tissue damage (41%; $p<.05$) compared to normothermic animals. The results support the conclusion that moderate, whole-body hypothermia is neuroprotective following acute traumatic SCI. Supported by NS30291 (WDD) and U.S. Army (RPY).

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